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Spike Protein VP8* of Human Rotavirus Recognizes Histo-Blood Group Antigens in a Type-Specific Manner

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Rotaviruses (RVs), an important cause of severe diarrhea in children, have been found to recognize sialic acid as receptors for host cell attachment. While a few animal RVs (of P[1], P[2], P[3], and P[7]) are sialidase sensitive, human RVs and the majority of animal RVs are sialidase insensitive. In this study, we demonstrated that the surface spike protein VP8* of the major P genotypes of human RVs interacts with the secretor histo-blood group antigens (HBGAs). Strains of the P[4] and P[8] genotypes shared reactivity with the common antigens of Lewis b (Le^b) and H type 1, while strains of the P[6] genotype bound the H type 1 antigen only. The bindings between recombinant VP8* and human saliva, milk, or synthetic HBGA oligosaccharides were demonstrated, which was confirmed by blockade of the bindings by monoclonal antibodies (MAbs) specific to Le^b and/or H type 1. In addition, specific binding activities were observed when triple-layered particles of a P[8] (Wa) RV were tested. Our results suggest that the spike protein VP8* of RVs is involved in the recognition of human HBGAs that may function as ligands or receptors for RV attachment to host cells.

Rotaviruses (RVs) are the most important cause of severe gastroenteritis in children. As a member of the reovirus family, RV has an icosahedral structure that consists of three concentric protein layers surrounding the double-stranded-RNA genome. To initiate an infection, the virion must pass through the host cell membrane by attachment to a cellular receptor, followed by delivery of its double-layered subviral particle into the cytoplasm (2, 16, 18, 23, 28, 42, 52, 53). Two major structural proteins, VP4 and VP7, are found on the outmost surfaces of the virions (11, 37). VP7 is a glycoprotein found rich in the endoplasmic reticulum of the infected cells and is mainly involved in virion assembly, while VP4 forms the major spike protein responsible for viral attachment and penetration into host cells (17, 28, 46, 47).

VP4 is processed by proteolytic cleavage into two subunits, VP5* and VP8* (14, 44, 45). VP8* is believed to be mainly involved in the attachment of viruses to host cells and VP5* in the translocation of the double-shelled particles into the cytoplasm through conformation rearrangement and membrane fusion (2, 14, 43), although the precise functions of the two subunits remain to be defined. Some RV strains recognize the terminal *N*-acetyl neuraminic (sialic) acid (SA) residues of carbohydrates on the host cell surface for attachment (15, 63, 64). However, this interaction appears nonessential for other strains (9, 41). RV-SA recognition has relied mainly on observation of neuraminidase sensitivity tests previously (36, 65), in which a strain was considered sialidase sensitive if its infection is sensitive to neuraminidase treatment. Recent reports showed that neuraminidase is capable of removing the terminal SAs without affecting subterminal SAs, and the infectivity and cell binding of two "sialidase-insensitive" RVs (Wa and DS-1) were increased following treatment of the host cells with neuraminidase (20, 27). Thus, the relative roles of the terminal versus the subterminal SAs in infection of RVs, particularly of the "sialidase-insensitive" human and animal RVs, remain unknown.

The sensitivity of RVs to sialidase treatment is associated with the VP8* sequence of P genotypes but not with their host origin, suggesting a linkage between the protein VP8* and viral attachment to host cells (9). RVs of P[1], P[2], P[3], and P[7] genotypes can be found in both humans and animals, and these RVs are mainly sialidase sensitive (9). Three P types (P[4], P[6], and P[8]) have been found to commonly cause gastroenteritis in humans, but none of them are sialidase sensitive (8). Thus, we hypothesize that these P types of human RVs may recognize an alternative carbohydrate, such as the human histo-blood group antigen (HBGAs), similarly to human noroviruses (NoVs) (57).

HBGAs are complex carbohydrates present on the surfaces of red blood cells and mucosal epithelia of the respiratory, genitourinary, and digestive tracts (10, 19, 21, 22, 33). They are also present as free oligosaccharides in biologic fluids, such as saliva, intestinal contents, milk, and blood (38). The biosynthesis pathway of HBGA starts with a disaccharide precursor by sequential additions of monosaccharides catalyzed by glycosyltransferases encoded by three major gene families, the ABO, Lewis, and secretor families. Each of the gene families contains silent alleles, leading to null phenotypes of the loci. For example, the FUT2-inactivated mutations are responsible for the nonsecretor phenotype found in about 20% of European and North American populations. The nonsecretor phenotype is characterized by the absence of ABH antigens in saliva and on most epithelial cells of the respiratory, genitourinary, and digestive tracts.

Human NoVs recognize different host HBGAs of individuals with different genetic makeups of the ABO, secretor, and Lewis families. The major human blood types of secretor, A/B, and Lewis bloods are determined by three unique terminal saccharides. They are α-1,2-fucose, α-*N*-acetylgalactosamine/α-galactose, and α-1,

Received 23 June 2011 Accepted 7 February 2012
Published ahead of print 15 February 2012
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doi:10.1128/JVI.05507-11

TABLE 1 Primers used for amplification of the RV VP8* and VP8* core coding region

Primer[a]	Sequence	Restriction enzyme site	Strain (position)	P genotype(s) to be amplified	Sense
P541	5′-CGTGGATCCATGGCTTCGCTCATTTATAGACA	BamHI	Wa (10–32)	P[8], P[6], P[4]	Positive
P556	5′-AGTGTCGACTCAGTCATCTAGTATTTTGAATTGGTGGTA	SalI	Wa (699–721)	P[8], P[6], P[4]	Negative
P1182	5′-CGTGGATCCGCTCCAGTCAATTGGGGTCATGGA	BamHI	Wa (145–168)	P[8]	Positive
P1183	5′-CGTGGATCCGCTCCAGTTGATTGGGGACACGGA	BamHI	RV5 (145–168)	P[4]	Positive
P1184	5′-CGTGGATCCGCACCAGTGACTTGGAGTCATGGGG	BamHI	ST3 (145–168)	P[6]	Positive
P1185	5′-AGTGTCGACTCAGTCATCTAGTATTTTGAATTGGTGG	SalI	Wa (679–699)	P[8], P[6], P[4]	Negative
P1186	5′-AGTGTCGACTCAGTCACCTTGTATTCTGCATTGGTGGTA	SalI	US1205 (677–699)	P[6]	Negative
P1279	5′-ACAACTAGTTTAGATGGTCCTTATCAAC	SpeI	Wa (202–220)	P[8], P[4]	Positive
P1281	5′-GATATCGATTAGACCGTTGTTAATATATTCATTACA	ClaI	Wa (656–678)	P[8], P[4]	Negative
P1509	5′-ACAACTAGTCTCGATGGTCCTTATCAAC	SpeI	BM11596 (202–220)	P[6]	Positive
P1510	5′-GATATCGATTAACCCAGTATTTATGTATTCACTACA	ClaI	BM11596 (656–678)	P[6]	Negative
P1504	5′-CGTGTCGACATGGCTTCGCTCATTTATAGACA	SalI	Wa (10–32)	P[8], P[6], P[4]	Positive
P1505	5′-GTGCGGCCGCTCAGTCATCTAGTATTTTGAATTGGTGGTA	NotI	Wa (699–721)	P[8], P[6], P[4]	Negative

[a] For expression of VP8*-GST fusion proteins, primer pair P541-P556 was used for amplification of VP8*, and primer pairs P1182-P1185, P1183-P1185, and P1184-P1186 were used for amplification of VP8* core. For expression of VP8* presented by the NoV P particle, primer pairs P1279-P1281 and P1509-P1510 were used for amplification of VP8* core. For expression of the VP8*-NoV S domain fusion protein, primer pair P1504-P1505 was used for amplification of VP8*.

3/4-fucose, respectively. These three saccharides have been shown to play an important role in binding to NoVs (57). SA is another residue that commonly occurs on mucosal surfaces, and this residue has been suggested to be involved in binding to some NoVs (48, 60). We hypothesize that RVs could share common carbohydrates as receptors with NoVs because RVs and NoVs may infect the same enterocytes in the intestinal tract.

Interaction of human RV with a carbohydrate has also been suggested by a crystallographic study of RV VP8* proteins of a sialidase-sensitive animal strain (CRW-8) and a sialidase-insensitive human strain (Wa) (7). A comparison of these two atomic structures showed that the SA binding pocket is missing for the sialidase-insensitive strain Wa. Instead, the crystal structure of WA VP8* revealed a novel groove region that is suggested to interact with carbohydrate (7). In addition, this groove is found to be conserved in both the sialidase-insensitive human strains and the sialidase-sensitive animal strains (7). Similar findings were reported in a separate study comparing the RRV (sialidase-sensitive) and DS-1 (sialidase-insensitive) VP8* proteins, in which a similar surface cleft was found on the DS-1 VP8* protein (43).

In this study, we provided the first evidence of interaction between human RVs and HBGAs in human saliva and milk. The results were further validated by binding of VP8* to synthetic oligosaccharides representing specific HBGAs and by blocking the binding by monoclonal antibodies (MAbs) specific to these HBGAs. More importantly, we observed similar binding activities with the use of authentic RVs recovered from cell cultures. While direct evidence is still needed, our results suggest that the sialidase-insensitive human RVs may recognize human HBGAs as ligands or receptors.

MATERIALS AND METHODS

Virus strains and primers. Different VP4 genotypes of human RVs, including cell culture-adapted RVs {Wa (P[8]) (GenBank accession no. M96825), RVP (P[8]) (GenBank accession no. EF672598), DS1 (P[4]) (GenBank accession no. EF672577), and ST3 (P[6]) (GenBank accession no. L33895)} and RV strains directly isolated from stool samples {BM13851 (P[8]), BM14113 (P[8]), BM151 (P[8]), BM5265 (P[4]), and BM11596 (P[6])}, were studied. A set of primers was designed (Table 1) to amplify the VP8*- and VP8* core (deletion of the first 64 amino acids)-encoding cDNA fragments by PCR. Viral RNAs were extracted either from cell culture or from clinic stool samples.

Cloning, expression, and purification of VP8*. The cDNAs encoding RV VP8* or VP8* core were cloned into the expression vector pGEX-4T-1 (glutathione *S*-transferase [GST]–gene fusion system; GE Healthcare Life Sciences, Piscataway, NJ) at the SalI and BamHI sites; after sequence confirmation, full-length VP8* and VP8* core fusion

TABLE 2 Panel of synthetic oligosaccharides used in detection of RV VP8*-oligosaccharide interactions

Oligosaccharide conjugate	Source[a]
Le^a-LC-LC	CFG
H type 1-LC-LC	CFG
H type 2-LC-LC	CFG
Le^y-LC-LC	CFG
TriLe^x-LC-LC	CFG
H type 2-LC-LC	CFG
A tetra type 2-LC-LC	CFG
B tetra type 2-LC-LC	CFG
B tetra type 1-LC-LC	CFG
A tetra type 1-LC-LC	CFG
Le^x-LC-LC	CFG
Le^x-PAA (30 kDa)	CFG
Le^x-PAA (1 MDa)	CFG
H type1-PAA (30 kDa)	CFG
H type1-PAA (1 MDa)	CFG
3′SLe^a-PAA (1 MDa)	CFG
3′SLe^x-PAA (1 MDa)	CFG
Tri-Le^x-PAA (30 kDa)	CFG
Tri-Le^x-PAA (1 MDa)	CFG
Le^a-PAA	GlycoTech, Inc.
Le^b-PAA	GlycoTech, Inc.
Le^x-PAA	GlycoTech, Inc.
Le^y-PAA	GlycoTech, Inc.
H type 1-PAA	GlycoTech, Inc.
H type 2-PAA	GlycoTech, Inc.
H type 3-PAA	GlycoTech, Inc.
Adi-PAA	GlycoTech, Inc.
Bdi-PAA	GlycoTech, Inc.
Atri-PAA	GlycoTech, Inc.
Btri-PAA	GlycoTech, Inc.
SLe^a-PAA	GlycoTech, Inc.
SLe^x-PAA	GlycoTech, Inc.

[a] CFG, Consortium for Functional Glycomics (a large research initiative funded by NIGMS to define the paradigms by which protein-carbohydrate interactions mediate cell communication [https://www.functionalglycomics.org]).

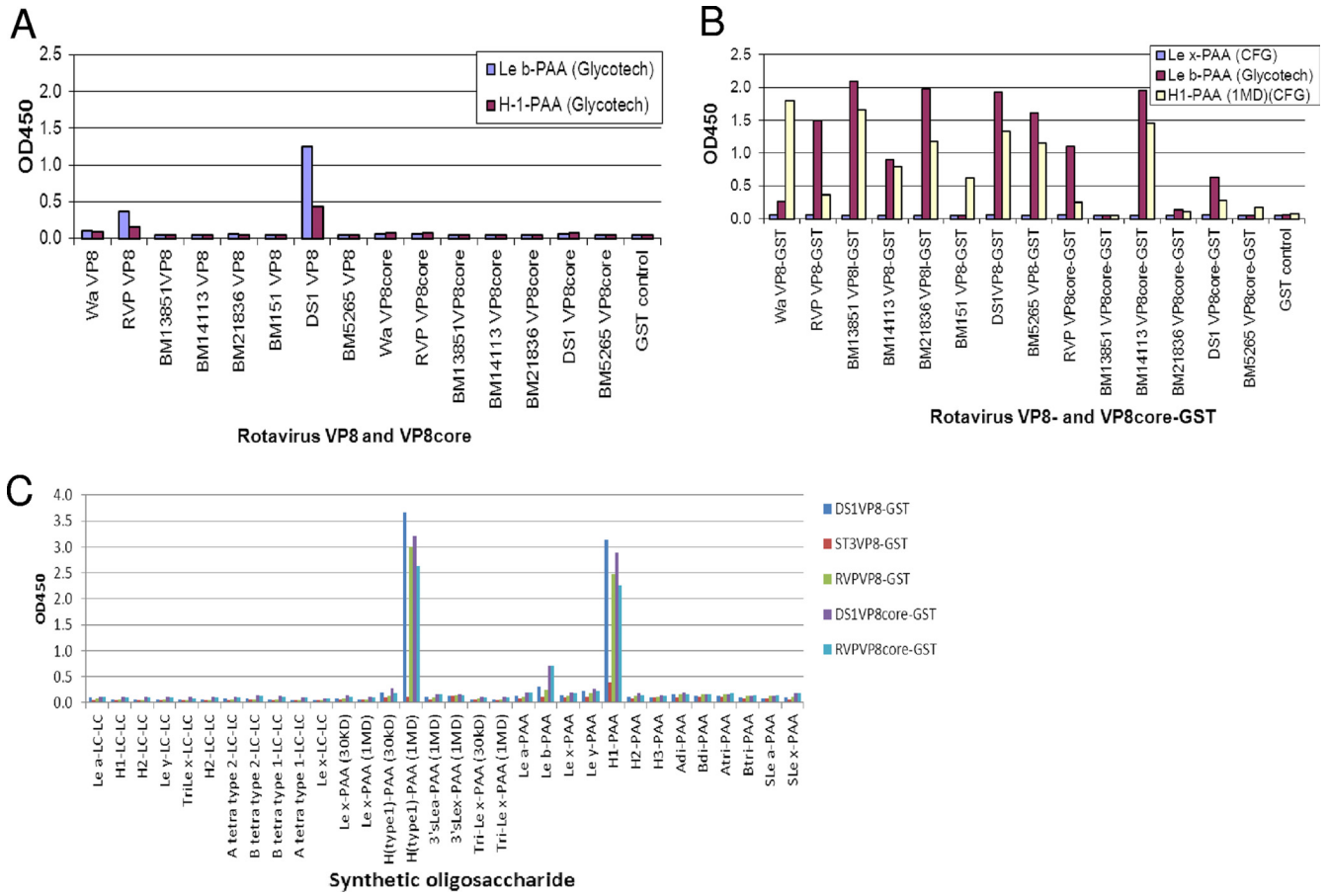

FIG 1 Binding of recombinant RV VP8* to synthetic oligosaccharides. Recombinant proteins with the full-length or the core region of the RV VP8* from seven P[8] or P[4] human RVs were tested for binding to oligosaccharides with (A) or without (B) removal of the GST tag. Except for BM13851 VP8* core-GST to H type 1-PAA and BM13851 VP8* core-GST, BM151 VP8*-GST, and BM5265 VP8* core-GST to Le^b-PAA, all VP8*- and VP8* core-GST proteins showed significant binding to Le^b and H type 1 antigens compared with binding to Le^x-PAA (means + 2 standard deviations [SD]). (C) One of each of the P[4] (DS1) and P[8] (RVP) RVs was tested with extended oligosaccharides representing variable HBGAs from two different sources (GlycoTech Corp. and the CFG) to confirm the binding specificities to the Le^b and H type 1 antigens.

proteins were expressed in *Escherichia coli* strain BL21 as described previously (55, 58, 59). For presentation of RV VP8* on the P particle of a genogroup II, genotype 4 (GII.4) norovirus (VA387), the P particle vector with a cloning cassette constructed previously (56) was used for cloning RV VP8* core into loop 2 of the P particle. For presentation of RV VP8* by the S protein, RV VP8* was fused to the C terminus of the shell (S) domain of VA387, which was cloned into the pGEX-4T-1 GST expression vector. Briefly, the expressions were induced by IPTG (isopropyl-β-D-thiogalactopyranoside; 0.4 mM) at room temperature (~22°C) overnight. RV VP8*-GST fusion protein was purified using glutathione Sepharose 4 fast flow (GE Healthcare Life Sciences) according to the manufacturer's protocol. The free VP8*, VP8*-P domain, and VP8*-VA387 S domain fusion proteins were released from GST by thrombin (GE Healthcare Life Sciences, Piscataway, NJ) digestion at room temperature overnight. The possible high-molecular-weight (MW) complex formation was examined by using size exclusion gel filtration (Superdex 200; GE Healthcare Life Sciences, Piscataway, NJ).

Testing of RV VP8* attachment to carbohydrates in human saliva by EIA. The saliva samples used in these studies were selected from a set of well-defined saliva samples previously described (24, 25). Saliva binding enzyme immune assays (EIA) were used to detect binding of recombinant VP8*/VP8* core to carbohydrates in saliva as described previously (24, 25). Boiled saliva samples were diluted at 1:1,000 and coated onto 96-well microtiter plates (Dynex Immulon; Dynatech, Franklin, MA) at 4°C overnight. After blocking with 5% nonfat milk, free VP8* or VP8* fusion proteins at 5 to 15 μg/ml or as indicated were added and incubated at 37°C for 1 h. The bound VP8* proteins were detected using guinea pig serum anti-BM151 VP8* at 1:5,000, followed by addition of horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (ICN, Aurora, OH). The signal intensities were displayed by a TMB kit (Kierkegaard and Perry Laboratory, Gaithersburg, MD), and then the optical density (OD) at 450 nm was read using an EIA spectra reader (Tecan, Durham, NC). In each step, the plates were incubated for 1 h at 37°C and washed five times with phosphate-buffered saline (PBS)–Tween 20 using a plate washer.

Testing of RV VP8* attachment to synthetic oligosaccharides. For the oligosaccharide-based binding assays, microtiter plates were coated with recombinant VP8* at 5 to 15 μg/ml at 4°C overnight or 37°C for 1 h as indicated. After blocking with 5% Blotto nonfat milk, oligosaccharide-polyacrylamide (PAA)-biotin conjugates (2 μg/ml) or oligosaccharide-6-aminohexanoate (LC)–biotin (2 μg/ml) was incubated at 4°C overnight. Bound oligosaccharides were detected using HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and displayed using the TMB kit as described above. A panel of oligosaccharide-PAA/LC-LC-biotin conjugates (Table 2) from two resources (GlycoTech Corporation,

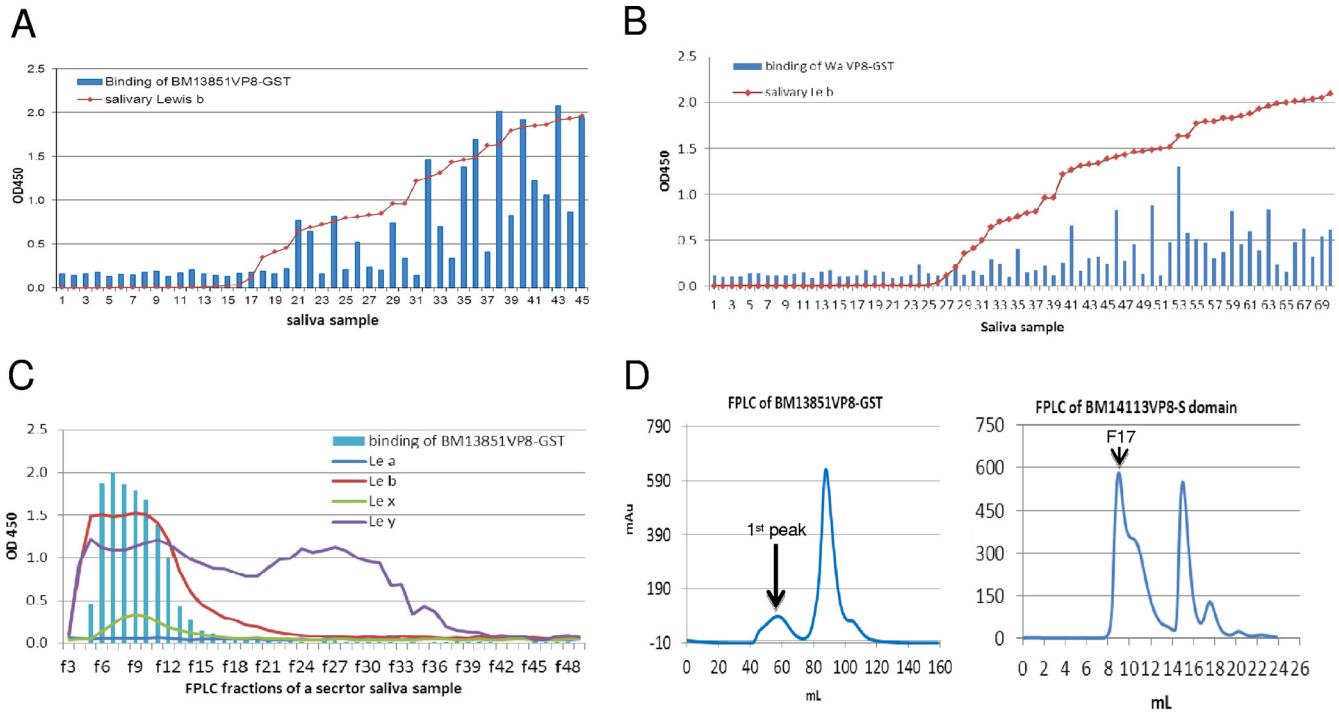

FIG 2 Binding of recombinant RV VP8* to saliva. (A and B) VP8*-GST fusion proteins from 2 P[8] (BM13851, Wa) human RVs were tested for binding to a panel of saliva samples. The results for binding of VP8* to individual saliva samples were plotted according to a sorting of the Le^b signals of individual saliva samples. Saliva samples were boiled before being used in the assays to remove antibodies fractionation by may interfere with the binding results. A correlation between the salivary Le^b and VP8* binding levels for both strains was observed. (C) Binding of an RV VP8* (BM13851) to a secretor saliva sample following the fast-performance liquid chromatography (FPLC). The signals of Le^a, Le^b, Le^x, and Le^y of individual fractions were determined by a MAb-based EIA. The VP8*-GST fusion protein bound only to the high-molecular-weight fractions containing the Le^b antigen. The chart was made by sorting of binding signals that were presented in line with markers instead of bars indicating correlation with the salivary Lewis antigens. (D) Gel filtration profiles of RV VP8*-GST (Superdex 200 16/60 GL) and VP8*-VA387 S domain fusion proteins (Superdex 200 10/300 GL). The molecular masses of the 1st peak of BM13851 VP8*-GST and fraction 17 (F17) of the BM14113 VP8* S domain were >440 kDa and >800 kDa, respectively.

Rockville, MD, and Consortium for Functional Glycomics [CFG]) was used for screening possible interaction.

Blocking of binding of RV VP8* to saliva and oligosaccharides by MAbs. The same conditions and procedure of saliva binding assays as those described above were used with an additional blocking step, in which saliva-coated plates were preincubated with MAbs specific to Le^a, Le^b, Le^x, Le^y, H type 1, and H type 2 at a dilution of 1:20 for 1 h at 37°C, before addition of recombinant VP8* to the plate. The blocking levels (%) were calculated by comparing the OD_{450} values between wells with or without incubation with a MAb.

Preparation of RV DLPs and TLPs by CsCl gradient centrifugation. A cell culture-adapted P[8] RV (Wa) was studied. A total of 1.0 liter of Wa-infected MA 104 cell cultures from 12 rolling bottles was harvested. After three cycles of freeze-thawing of the samples and clarification by a low-speed centrifugation, the viruses in the supernatants were concentrated by pelleting them at 28,000 rpm for 3 h using a SW28 rotor (Beckman). The viruses in the pellets were resuspended in TNC buffer (50 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, pH 7.5) containing CsCl. The final density of the sample was adjusted to a refractive index of 1.369 before the sample was loaded into the rotor. After centrifugation at 288,000 × *g* for 46 h using a SW41Ti rotor (Beckman), the gradient was fractioned by bottom puncture and ~0.5-ml fractions were collected. The fractions containing double-layered particles (DLPs) or triple-layered particles (TLPs) were identified based on the densities and then dialyzed against TNC buffer. The resulting DLP and TLP preps were examined by transmission electron microscopy (TEM) using negative staining and further confirmed by Western blot analysis using a VP8*-specific MAb. The DLP and TLP preps were then quantified by SDS-PAGE based on the extensity of the major VP6 protein on the gels, and equal amounts of viruses were used to determine the cell culture infectivity.

Binding of CsCl gradient-purified DLPs and TLPs to saliva samples. Procedures similar to those for the saliva binding assays described above were used. Briefly, boiled saliva samples were diluted at 1:1,000 and coated onto 96-well microtiter plates at 4°C overnight. After blocking with 5% nonfat milk, fractions 7 and 10, which contain DLPs and TLPs, respectively, from the Wa CsCl gradient were added at 1:10 with 1% nonfat milk. After an overnight incubation at 4°C, the binding signals were detected by the HRP-antibody enzyme conjugate from the Rotaclone kit (Meridian Biosciences, Cincinnati, OH).

Research using clinical specimens. Human saliva and milk samples used in this study were archived samples from previous studies (24, 25, 29) approved by the institutional review board (IRB) of the Cincinnati Children's Hospital Medical Center.

RESULTS

The VP8* proteins of P[8] and P[4] RVs recognize the Le^b and/or H type 1 antigens. We initiated our study by performing saliva- and oligosaccharide-based binding assays using recombinant RV spike proteins made from *E. coli* cultures. When highly purified VP8* proteins from a number of P[4] and P[8] RVs were examined, the signals for binding to saliva were very weak and not stable (data not shown), but binding activities with synthetic oligosaccharides of Le^b and H type 1 antigens were clearly observed for two strains of P[8] and P[4], respectively (Fig. 1A). Notably, the binding signals increased significantly when the GST-VP8* fusion pro-

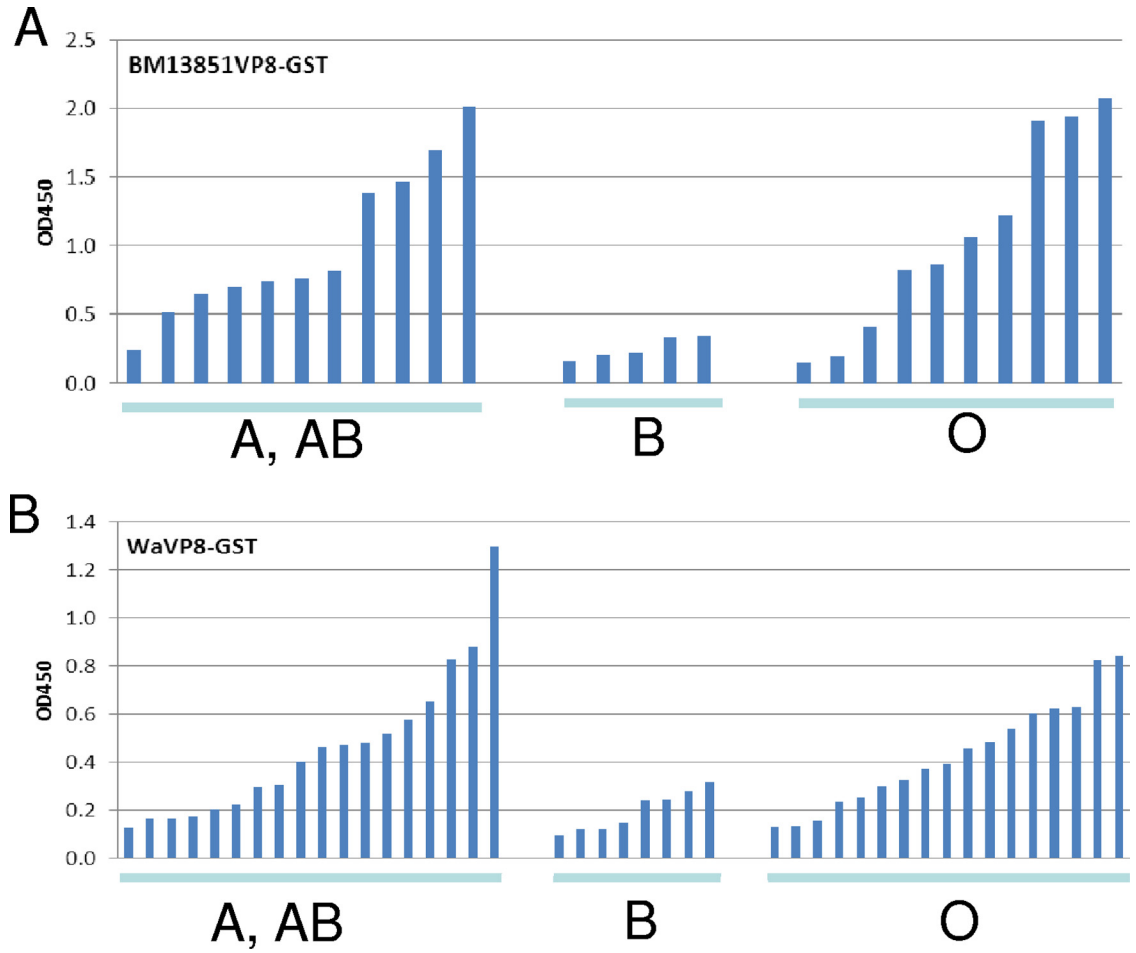

FIG 3 Characterization of saliva binding signals of RV VP8* based on ABO typing of saliva. The signals for binding of VP8*-GST of BM13851 (A) and Wa (B) to saliva from type A/AB, B, and O secretors (Le^b positive) were compared. The low-level binding of VP8* to type B secretor saliva may due to a steric interference from B antigen. The individuals in each of the three groups were sorted from low to high binding strength (OD).

teins were tested (Fig. 1B and C). GST fusion of VP8* from 2 P[4] and 5 P[8] strains, including RVs isolated directly from stool specimens and cell culture-adapted strains (Wa, DS-1, and RVP), revealed similar binding profiles for Le^b and H type 1 antigens (Fig. 1B). The binding activities were from VP8*, not from the GST tag, because purified GST proteins alone did not reveal any binding activity (Fig. 1B). We also performed binding assays on extended HBGA oligosaccharides with selected P[4] and P[8] strains; among a panel of 32 oligosaccharides from different sources tested, only the Le^b and H type 1 antigens revealed strong binding activities (Fig. 1C).

The oligosaccharide binding results for the GST-VP8* fusion proteins were confirmed by the saliva-based binding assays, in which a strong association of binding signals for 2 selected P[8] strains with the salivary Le^b antigen were detected using a panel of well-characterized saliva samples (Fig. 2A and B). The relationship of binding of VP8* to H type 1 was difficult to establish due to the low signal intensity of H type 1 in most of the saliva samples tested, particularly in Le^b-positive individuals, because H type 1 is the precursor of Le^b. The products derived from the type 2 precursor may not be involved, because no correlation of Le^y with VP8* binding was identified (Fig. 2C). In addition, we noticed an influence of binding by the type B epitope. Among the Le^b-positive individuals, the signals for binding to the type B saliva were significantly lower than those for binding to the type A/AB and O saliva for both of the P[8] strains studied (Fig. 3), suggesting that the terminal α-galactose (the type B epitope) may interfere with the binding by masking the H epitope (α 1,2-fucose), the major determinant of the secretor type. No such interference by the type A epitope was observed.

The binding of VP8* was enhanced by fusion to the shell (S) or protruding (P) domains of NoV capsid protein. In our separate studies, we found that the norovirus (NoV) capsid protein can be used as an excellent scaffold for presentation of foreign antigens for immune enhancement (57). To further verify the HBGA binding activities of VP8* described above, recombinant chimeric NoV P particles with VP8* proteins inserted in one of the surface loops of the P protein were constructed. In addition, VP8* fusion proteins fused to the C terminus of the NoV S protein were developed. The VP8* antigens were well presented by both P and S proteins; 24 copies of VP8* were present on each P particle (57), and high-molecular-weight complexes of the S-VP8* fusion proteins were detected by gel filtration analysis (Fig. 2D). High binding activities with minor variations compared with the activities of the GST-presented VP8* protein were observed (Fig. 4). The S-VP8* fusion proteins revealed binding to both the Le^b and the H

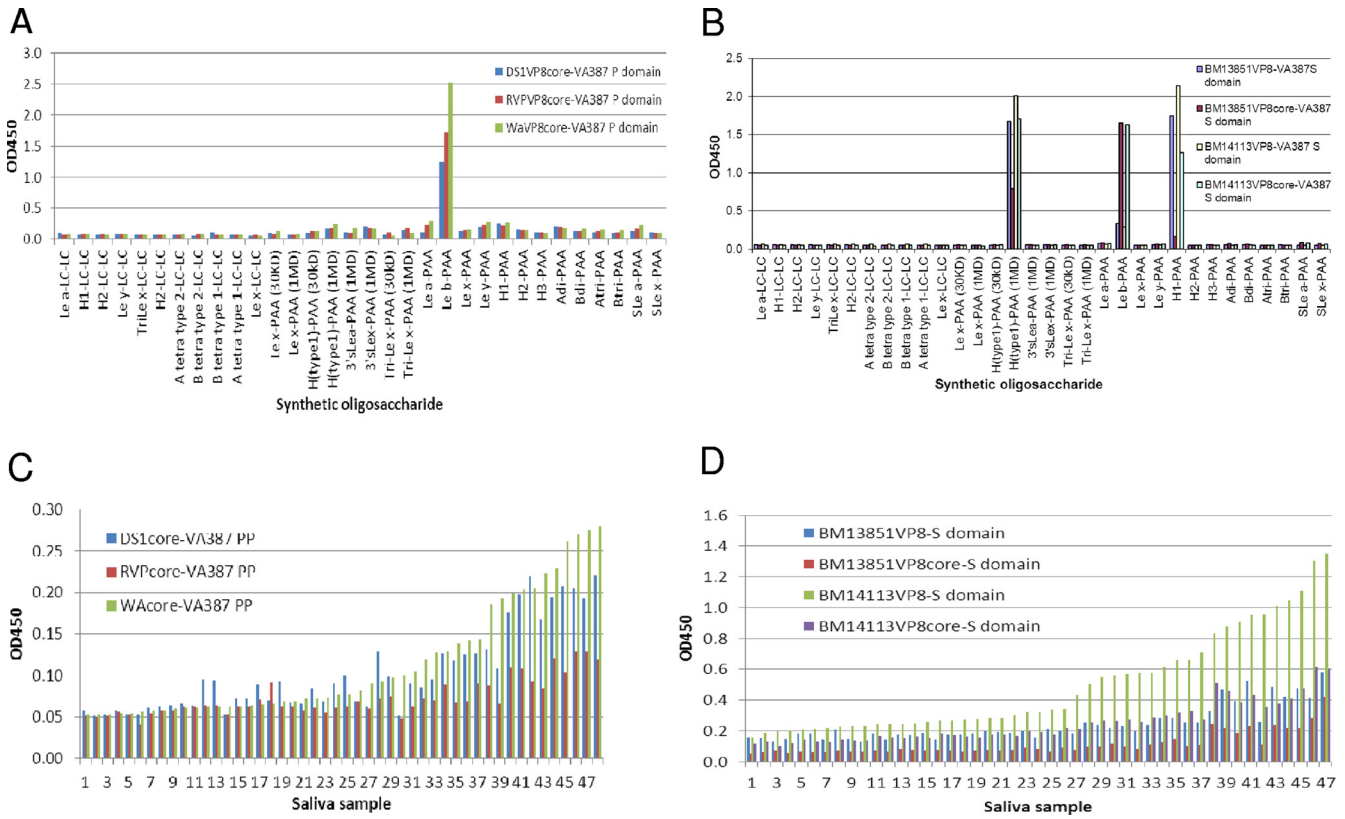

FIG 4 Binding of RV VP8* presented by the NoV VA387 P particle (PP, P domain) and the S complex to synthetic oligosaccharides and saliva. (A) VP8* presented by the NoV P particle bound to the Le^b oligosaccharides for both P[8] (Wa and DS1) and P[4] (RVP) RVs. (B) VP8* presented by the NoV S domain bound to the H type 1 in addition to the Le^b antigens. (C and D) The bindings of VP8* to a panel of Le^b-positive saliva samples were correlated with each other among the VP8* proteins presented by the NoV P particle (C) or the S domain (D).

type 1 oligosaccharides that was identical to that of the VP8*-GST fusion proteins, although the P particle-presented VP8* protein mainly bound to the Le^b oligosaccharide (Fig. 4A and B). The saliva binding patterns were more consistent: both the P particle- and the S protein-presented VP8* proteins bound specifically to the Le^b-positive secretors, with similar strain variations among a panel of saliva samples studied (Fig. 4C and D).

The VP8* protein of P[6] RV recognizes the H type 1 antigen only. We then extended the study to another major P type, P[6], of human RVs. Similar low binding activities of the free VP8* proteins (data not shown) and enhanced binding signals of the GST- and P particle-presented VP8* proteins were observed for two P[6] strains (BM11596 and ST3) studied. The binding patterns of the P[6] strains, however, differed from those of the P[4] and P[8] strains; the two P[6] strains bind the H type 1 antigen only without binding to the Le^b antigen (Fig. 5). Surprisingly, the P[6] VP8* protein revealed only marginal binding activities with saliva samples even when the GST- and P particle-presented VP8* proteins of the two strains were tested (data not shown). To verify the result, a panel of Le^b-negative saliva samples was studied. Although the binding signals were still low, a correlation with binding of VP8* to the salivary H type 1 antigen was noticed (Fig. 5C).

Validation of the binding specificity by HBGA-specific MAbs. To further validate the binding specificity, we measured blocking effects of MAbs specific to variable HBGAs on the binding of the GST-VP8* fusion proteins to saliva and oligosaccharides. Only MAb specific to Le^b but not to other HBGAs blocked binding of GST-VP8* of P[8] to synthetic oligosaccharide (Fig. 6A). The blockade of binding to saliva, however, seemed variable; MAbs against Le^y and H type 2 in addition to Le^b blocked binding of GST-VP8* to saliva (Fig. 6B). It is known that human saliva usually contains a mixture of different HBGAs, including ABO, Lewis, and secretor types, of which Le^b, Le^y, and H type 2 contain common α1,2-linked fucose that may complicate the experiment outcomes.

RV-VP8* also recognizes Le^b in human milk. In study of a panel of human milk samples, we observed specific binding of P[8] VP8* to secretor-positive milk (data not shown). We then performed binding assays using two milk samples following separation of the components in the milk samples through gel filtration. Again, the P[8] VP8* protein bound to the Le^b-positive but not the Le^b-negative milk sample (Fig. 7 and data not shown). Interestingly, the binding signals were mainly detected in fractions containing the high-molecular-weight (MW) molecules, similar to that observed in binding to NoVs that recognize only the high-MW mucin or mucin-like glycans (26).

RV triple-layered particles bound HBGAs. To further determine the biological relevance of the observed VP8*-HBGA interaction, virions of strain Wa were purified from cell cultures through a standard CsCl gradient. Evidence of double-layered particles (DLPs) and triple-layered particles (TLPs) was obtained by observation of typical DLPs and TLPs by electron microscopy (EM), by detection of additional VP7 and VP8* proteins in the TLP fraction but not in the DLP fraction by SDS-PAGE and West-

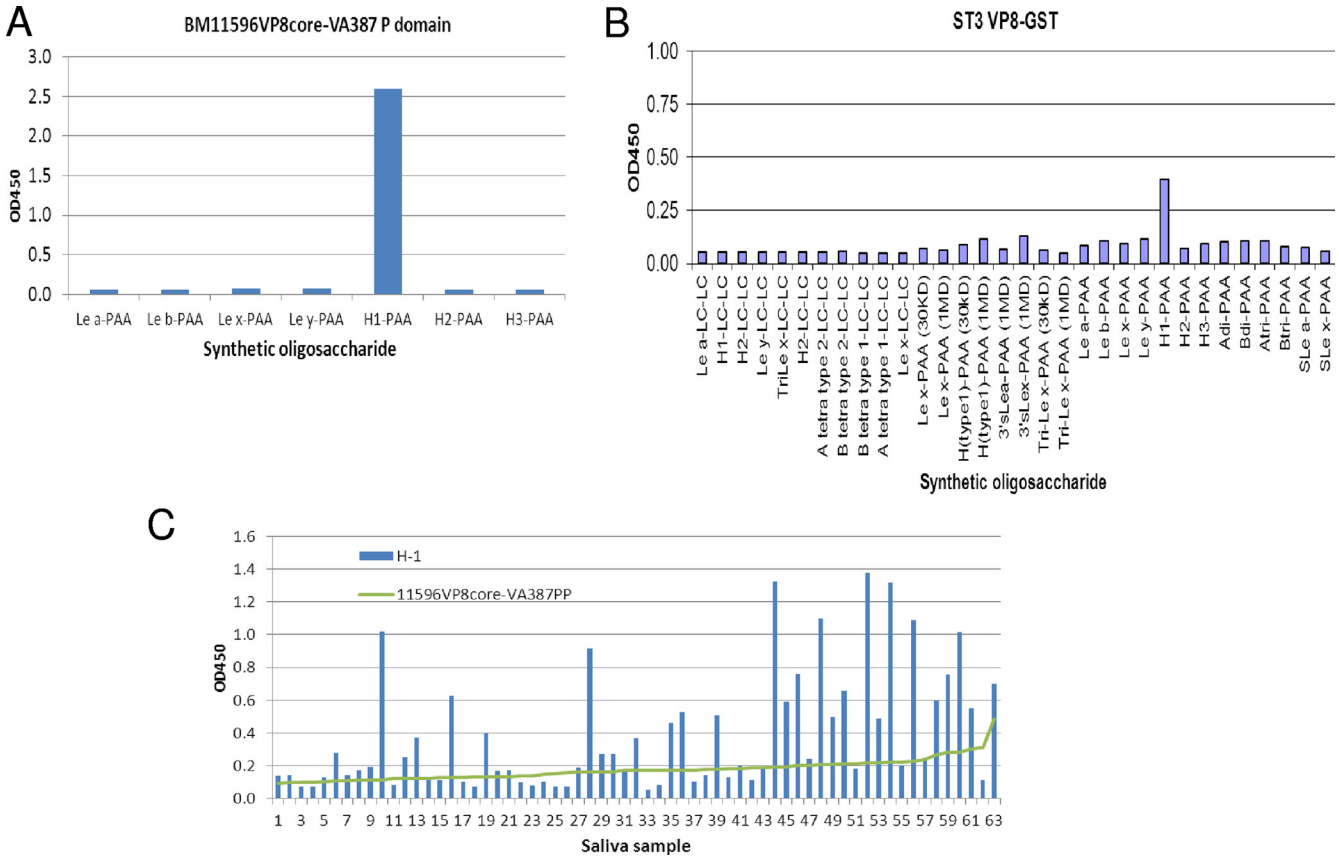

FIG 5 Binding of VP8* from two P[6] RVs to synthetic oligosaccharides. The ST3 VP8*-GST fusion protein and the BM11596 VP8* core protein presented by the VA387 P domain (P particle, PP) were tested by the oligosaccharide binding assays described in Materials and Methods. Both BM11596 (A) and ST3 (B) recognized PAA-conjugated H type 1 only. (C) A panel of Lewis b-negative saliva samples was tested for binding to BM11596 VP8*. The data for individual saliva samples were sorted by their binding activities with the VP8* protein.

ern blot analyses (Fig. 8), and by significantly higher (~256 times) infectivity of the TLP fraction than of the DLP fraction. The highly purified Wa TLPs revealed specific binding to Le^b-positive but not to Le^b-negative secretor saliva samples (Fig. 8). In contrast, the CsCl gradient fraction containing DLPs did not reveal such binding activity with either the Le^b-positive or the Le^b-negative saliva samples, even when 4-fold more DLPs than TLPs were used. Our results suggest that HGBA binding ability is the authentic nature of RVs, and the spike protein VP8* on the outer layer of RVs is likely to be responsible for the binding function.

DISCUSSION

In this study, we provided the first evidence of specific interactions between human RVs and HBGAs through the outer-layer spike protein VP8*. Two genetically closely related P genotypes (P[4] and P[8]) recognize the common Le^b and H type 1 antigens, while a genetically more distantly related P genotype (P[6]) recognizes H type 1 only. These binding activities were confirmed by different forms of recombinant VP8*. The binding specificities have also been verified by binding and blocking experiments using saliva, synthetic oligosaccharide, human milk, and MAbs specific to variable HBGAs. Finally, the biological relevance of the observed VP8*-HBGA interaction was shown by the binding of authentic RVs to the Le^b-positive saliva samples. These data suggested that the major P genotypes of RVs causing human acute gastroenteritis may recognize human HBGAs on host cells as attachment factors or receptors.

The requirement of a carbohydrate receptor appears to be a common feature of RVs. While some animal RVs have been shown to recognize the SA as receptors, our data in this study suggested that the human RVs may recognize the human HBGAs. This feature seems similar to what were found in caliciviruses. While the human caliciviruses, such as the human NoVs, recognize the human HBGAs, nonhuman caliciviruses, such as the murine NoVs and the feline calicivirus recognizing the SA as receptors, recognize other carbohydrates (51, 60). In a separate study, an additional HBGA binding pattern of RVs that recognizes the type A HBGAs by P[9] and P[14] RVs has been found (Y. Liu et al., submitted for publication). These data further suggested that the human HBGAs may play an important role in the infection of human RVs, although future studies for proving this hypothesis are necessary.

In the saliva- and oligosaccharide-based binding assays, we did not observe specific interaction of either P[4] or P[8] RVs with the SA-containing carbohydrate antigens, including sialyl Le^a and sialyl Le^x. This result is consistent with the notion that neither P[4] nor P[8] RVs recognize the terminal sialic acid (8, 9). However, whether any subterminal SAs on the host cells are involved in infection of these two genotypes remains unknown. Since the hypothesis of involvement of subterminal SAs in RV infection was

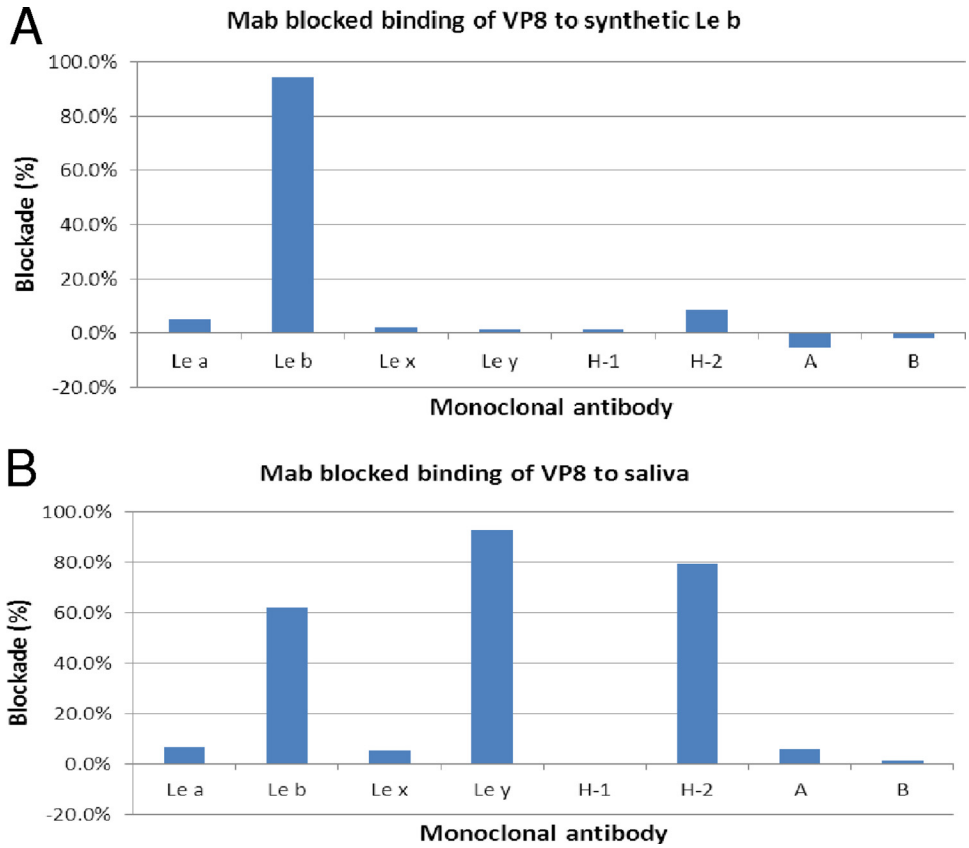

FIG 6 Blocking of binding of RV VP8* to synthetic oligosaccharide (A) and saliva (B) by monoclonal antibodies (MAbs) specific for HBGAs. Microtiter plates were coated with Le^b oligosaccharide (A) or Le^b-positive saliva (B) to capture RV VP8*-GST fusion protein (~10 μg/ml) with or without preincubation with MAbs (at a dilution of 1:20) against Le^a, Le^b, Le^x, Le^y, A, B, H type 1, and H type 2 antigens. VP8*-specific blocking activity was determined by the reduction (%) of the optical density values in wells with MAbs compared with those in wells without MAbs.

mainly based on increased infectivity and binding of RV Wa following the sialidase treatment of the host cells, there could be other epitopes in addition to SA that are also involved. Indeed, structural analyses of RV VP8* suggested that other carbohydrates may be involved in RV host interaction by observing an additional carbohydrate binding pocket on RV VP8* (7, 43). Thus, future studies for elucidating the role of subterminal SA as well as other carbohydrate epitopes in the infection of these "sialidase-insensitive" human RVs are necessary.

The finding that the P[4], P[6], and P[8] human RVs recognize the major secretor epitopes of human HBGAs appears to correlate with the predominance of these genotypes of RVs over other genotypes in causing disease in different populations. The α1,2-fucose is the major determinant of the secretor antigens that is syn-

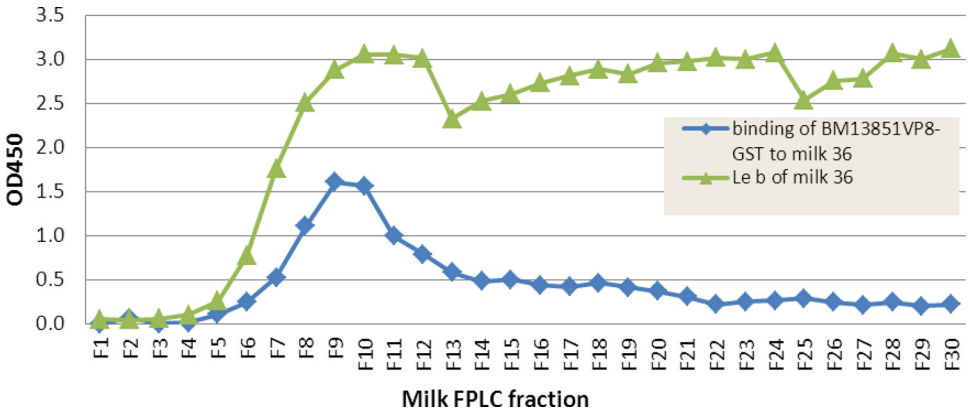

FIG 7 Binding of RV-VP8* to milk Le^b antigen in size-exclusive FPLC fractions. Boiled FPLC fractions of one secretor's milk sample (milk 36) were used to coat microtiter plates at a dilution of 1:15 in 1× PBS. The bound BM13851 VP8*-GST protein was detected by rotavirus hyperimmune sera. Only the high-molecular-weight fractions containing Le^b antigen showed binding. The distribution of milk Le^b in the FPLC fraction was previously measured in a separate experiment.

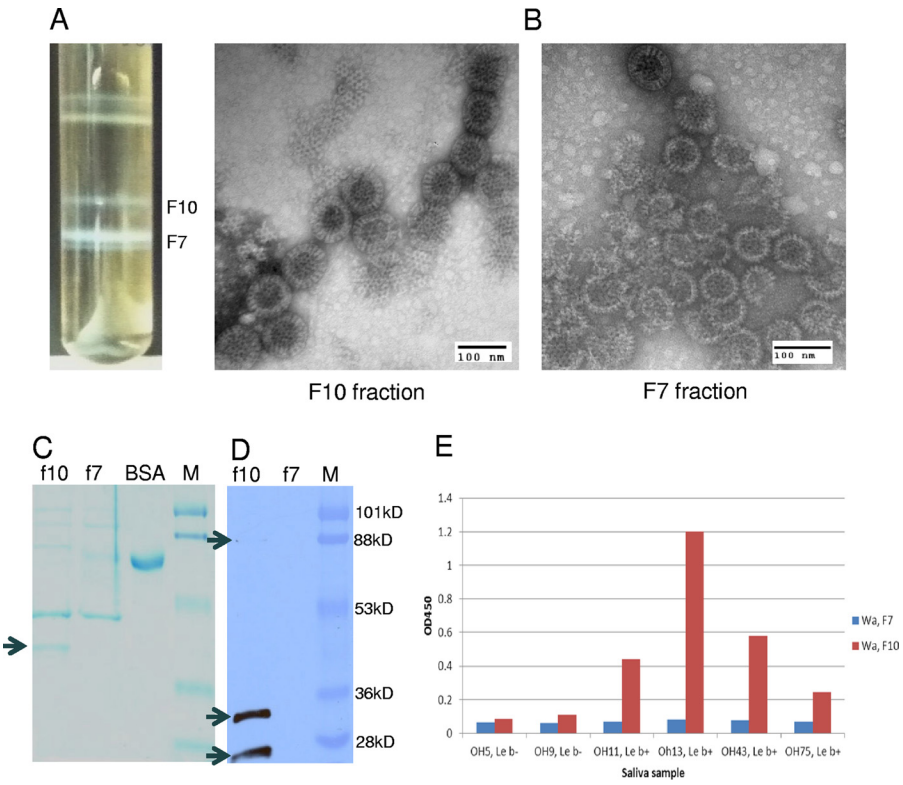

FIG 8 Purification of Wa TLP and its binding to saliva samples from Le^b-positive individuals. Wa TLP and DLP bands were observed in a CsCl gradient purification (A). DLPs (F7; density, 1.39 g/cm^3 in CsCl) and TLPs (F10; density, 1.36 g/cm^3 in CsCl) were confirmed by electron microscopy (EM) examination (B) and Western blot analysis (D). Typical TLPs and DLPs were observed in each of the two fractions by EM in negatively stained grids (B). SDS-PAGE followed by Coomassie blue staining revealed an extra protein band (38 kDa) (arrow) in F10 but not in F7, which is predicted to be the VP7 protein on the outer layer of TLPs, missing on DLPs. The amounts of viral loading of F10 and F7 in the gels were adjusted to equality on the basis of the amounts of VP6 in each of the two fractions (C). The presence of the other major outer layer surface protein VP4 was confirmed by detection of VP4 (top arrow), the protease-processed VP8* protein (middle arrow), and a truncated VP8* protein (bottom arrow) by Western blot analysis using a VP8*-specific monoclonal antibody (D). Specific binding of the Wa TLPs, but not the DLPs, to the Le^b HBGAs was demonstrated by the saliva-based binding assay using a panel of Le^b-positive and -negative saliva samples (E). Equal volumes (10 μl) of F7 and F10 were used in the saliva binding assay. A serial dilution analysis by SDS-PAGE showed that the amount of the DLPs was ~4-fold larger than that of the TLPs based on the intensities of the major structural protein VP6 in the two preps in the gel (data not shown).

thesized by the α1,2-fucosetransferase encoded by the *FUT2* gene. Secretor antigens are present in ~80% of the general population in the North American and European countries (33, 34) and possibly many other regions of the world as well. Thus, the high prevalence of these genotypes could be due to their interactions with wide spectrum of host HBGAs. In fact, >90% of the RV gastroenteritis cases were caused by P[8]- and P[4]-bearing RVs in many countries and for most years (13, 49). It is more interesting that the prevalences of the two P genotypes seemed always reciprocal to each other, with the P[8] genotype being predominant for most of years, according to surveillance data reported for Argentina, the United Kingdom, Australia, and Thailand (4–6, 13, 31, 32, 39, 40, 49). No such relationship was seen for P[6] RVs. These data suggested that the P[8] and P[4] RVs are competing with each other for commonly targeted populations of secretors.

The finding of P[6] RVs recognizing the H type 1 antigen also may explain their unique epidemic pattern. While much less common in many countries of the world, the P[6] RVs were significantly more common in the African countries (50, 54, 61, 62). In our previous studies of human HBGAs in different ethnic groups, the African-Americans appeared to have a significantly higher rate of the Lewis-negative secretor (H^+ Le^{a-b-}) phenotype than other ethnic groups (unpublished data). Genetically, these individuals do not have a functional Lewis gene and therefore can produce only H type 1, not Le^b. Since the P[6] RVs bind H type 1 only, the higher prevalence of P[6] RVs in the African countries could be due to a higher rate of the Le^{a-b-} phenotypes in these regions. However, high prevalence or endemicity of P[6] RVs has also been reported for India and for non-African newborn infants (3, 12, 30, 35). One report did not show association of secretor status with RV infection in children (1). Thus, future studies for clarifying these controversies by performing extended population studies in different countries and geographical locations are necessary.

In this study, we particularly studied recombinant VP8* proteins of RVs derived from stool samples of RV-infected patients. There was no difference between the HBGA binding profiles of these strains and those of cell culture-adapted strains, indicating that the observed HBGA binding was not an adapted property under the *in vitro* condition. We also studied multiple strains within each genotype and observed identical HBGA binding patterns among all strains within the same genotypes. In summary, the sialidase-insensitive human RVs may recognize the human HBGAs as an alternative attachment factor or as receptors, probably functionally similar to the terminal sialic acid for sialidase-sensitive animal RVs. The observed strain variations among different RV P genotypes suggest that further studies for exploring

additional variations and the roles of HBGAs in RV infection and evolution are warranted.

ACKNOWLEDGMENTS

We thank Dongsheng Zhang for valuable technical support and helpful discussion.

This study was supported by the National Institutes of Health, National Institute of Allergy and Infectious Diseases (R01 AI37093 and R01 AI055649), the National Institute of Child Health (P01 HD13021), and the Department of Defense (PR033018). This study was also supported by an NIH/NIAID grant (1R21AI092434-01A1) to M.T.

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